

Biosynthesis of the Two Chains of Insulin

with an addendum on

Partial Structural Analysis of Insulin from Separate Islet Tissue of Anglerfish (*Lophius piscatorius*)

by

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BIOSYNTHESIS OF THE TWO CHAINS OF INSULIN*

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The insulin molecule, consisting as it does of two polypeptide chains interconnected by two disulfide bridges,¹ presents a special problem in the elucidation of biosynthetic mechanisms. Chymotrypsin, a protein consisting of three polypeptide chains, is known to be derived from a single-chain precursor, chymotrypsinogen. Insulin might be produced in the same way, but no single-chain insulin precursor has as yet been found. Alternatively, the two constituent chains of insulin might be separately synthesized and subsequently connected by two disulfide bridges. It can be assumed that the correct formation of the five intrachain disulfide bridges in chymotrypsinogen occurs spontaneously, like the intrachain disulfide bridge formation in reduced ribonuclease and lysozyme.^{2, 3} For insulin, however, the situation seems to be different: it can be reconstituted *in vitro* from its reduced constituent chains with a yield of only 1-2 per cent.⁴

The purpose of the present work was to prove or rule out one of these two possibilities: (1) separate synthesis of the two constituent chains, with subsequent formation of the interchain disulfide bridges; (2) synthesis of a single-chain precursor.

sor having three intrachain disulfide bridges, with subsequent peptide bond hydrolysis to yield the two chains.

It was first assumed that biosynthesis of insulin, or of its hypothetical precursor, starts at its N-terminal amino acid residue(s) and proceeds linearly toward the C-terminal end(s), a pattern which has been found for a number of proteins (α -amylase,⁵ *E. coli* proteins,⁶ globin chains,⁷ egg white lysozyme,⁸ and ribonuclease⁹). It was further assumed that a short time incubation of insulin-synthesizing tissue with a specific labeled amino acid will yield a gradient of specific radioactivities along the chains, the lowest specific activity being in the part where synthesis starts, since a number of unfinished chains of varied length are expected to exist at the start of the incubation.

Considering the low specific activities of insulin which probably would be obtained by pulse labeling, the use of mammalian pancreas with its small percentage of insulin-synthesizing tissue was abandoned in favor of the Brockmann body of anglerfish (goosefish, *Lophius piscatorius*), an organ consisting of practically pure islet tissue.¹⁰ Previous work has established the proper conditions for *in vitro* studies of metabolic functions and of protein synthesis with isolated islet tissue of toadfish¹¹⁻¹³ and of anglerfish.^{14, 15}

A simple and reproducible method for isolation of insulin was devised,¹⁶ and the addition of a further purification step led to the isolation of pure insulin in good yield.¹⁷ Since the structure of this particular fish insulin was not known, the determination of its amino acid composition and partial structure was undertaken, as reported elsewhere.¹⁷ As a result, the positions of two of the three residues of proline in this molecule are known. These occur in the B chain, which corresponds to the phenylalanyl chain of beef insulin, at position B₂ and at B₂₈. The A chain, which corresponds to the beef glycyl chain, contains a single residue of proline,¹⁸ probably at position A₉ or A₁₀, by analogy with bonito insulin II.¹⁹

Thus, proline, occupying positions near the NH₂-terminus and COOH-terminus of the B chain, and a position most probably near the center of the A chain, is the amino acid whose incorporation was studied in the present experiments.

Experimental Procedures.—Incubation of islet tissue: Brockmann bodies from freshly caught anglerfish (*Lophius piscatorius*) were isolated directly on the fishing trawler (New England Biological Assn., Point Judith, R.I.). They were kept and shipped in Krebs-Ringer solution on ice. Time delay due to shipping averaged 6–10 hr, after which experiments were immediately started. Fifteen to twenty-five minced Brockmann bodies (totaling 1 gm wet weight) were incubated in 3–6 ml Krebs-Ringer bicarbonate buffer, pH 7.4, under 95% O₂/5% CO₂ in the presence of 0.5 mM radioactive L-proline (either L-proline-3,4-H³, 5 C/mM, or L-proline-C¹⁴, 200 mC/mM, New England Nuclear Corp. in a Dubnoff metabolic shaker at 17°C. Puromycin (Lederle Laboratories, Pearl River, N.Y.), when added, was dissolved in Krebs-Ringer buffer before addition to the medium. In experiments in which incorporation of proline into insulin and bulk protein was compared, the term "bulk protein" refers to material insoluble in cold 10% TCA and in 75% ethanol/0.18 N HCl. For the experiments designed to yield a gradient of radioactivity along the insulin chains, one batch of islet tissue was incubated with proline-H³ for 15–30 min, and another batch was incubated with proline-C¹⁴ for 5–8 hr. Incubation was stopped by adding TCA to 10% final concentration, whereupon the two batches were combined and processed together.

Isolation of insulin: Insulin extraction with acid ethanol from TCA precipitates and its purification on a column of Sephadex G-75 in 5 M acetic acid/0.15 M NaCl were performed as previously described.¹⁶ The final purification was achieved by column chromatography on CM-cellulose with a formic acid gradient.¹⁷

Isolation of peptides: Isolated insulin was reduced and carboxymethylated¹⁷ to yield the two chains which were subsequently treated with trypsin (Worthington) to split the only trypsin-susceptible bond Arg^{B22}-Gly^{B23}. The resulting three peptides (A chain, B₁₋₂₂, B₂₃₋₂₉), each containing one proline residue, were separated by paper electrophoresis in 0.2 M NH₄HCO₃, pH 8.1, at 20 v/cm for 3 hr and eluted in ascending fashion with 50% acetic acid.¹⁷

Isolation of proline: Each peptide was separately hydrolyzed in 6 N HCl in evacuated sealed tubes for 22 hr at 110°C. The hydrolysates were applied on the 50-cm column of an amino acid analyzer,²⁰ equipped with the accelerated system of Spackman.²¹ As an additional monitoring device, the column effluent was run through a 2-ml anthracene-packed flow-cell in a scintillation counter (Nuclear-Chicago), and was then fed back to the analyzer for the automatic ninhydrin reaction. This design allowed unequivocal identification as well as estimation of the purity of the isolated peptide, and, by counting the column effluent, provided a convenient means for immediately checking the exact position of proline in the effluent. The short counting time of 2 min in the flow counter was, however, too low to be of value in the calculation of H³/C¹⁴ ratios. The flow counter effluent containing the isolated proline (6 ml) was thus collected, desalted on a column (0.9 × 5 cm) of Dowex 1 × 8 (200-400 mesh, OH⁻ form) by eluting with 1 M acetic acid,²² and counted in a hyamine-toluene system in a two-channel liquid scintillation counter (Packard Tricarb). The settings of the counter were so chosen as to yield highest possible channel ratios for both C¹⁴ and H³. Efficiency for tritium counting in the first channel was 36%, for C¹⁴ in the second channel 57%. Calculations of C¹⁴ and H³ counts followed the procedure of Okita *et al.*²³ using C¹⁴- and H³-toluene as internal standards.

Results.—The time course of incorporation of proline-C¹⁴ into bulk protein and insulin is shown in Figure 1. Linear incorporation with time occurs only after the first hour of incubation. When slices were preincubated without labeled substrate

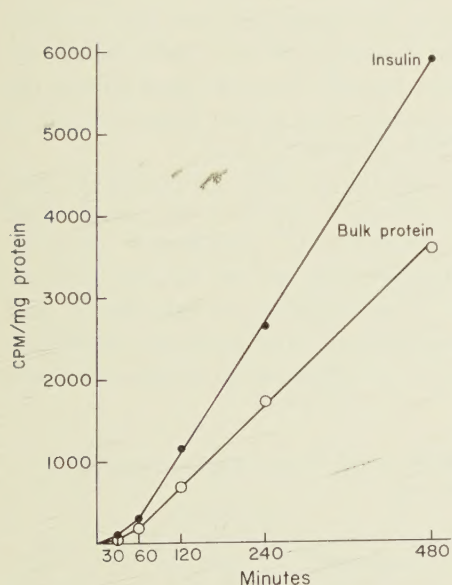


FIG. 1.—Time course of the incorporation reaction. Each flask contained about 250 mg wet weight of islet tissue minces in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with L-proline-C¹⁴, 200 μ C/ μ M, 0.5 μ M/ml. "Bulk protein" is the TCA- and 75% ethanol/0.18 N HCl-insoluble material. The purification of insulin was carried only to the Sephadex G-75 step, which leaves insulin contaminated with two peptides of small molecular weight and of low proline content.¹⁷

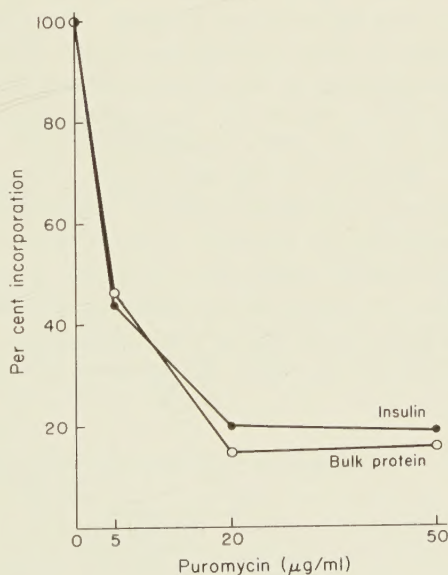


FIG. 2.—Effect of puromycin on the incorporation reaction. Results are expressed as per cent of control activity. Each flask contained about 300 mg wet weight of islet tissue minces in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with L-proline-C¹⁴, 200 μ C/ μ M, 0.5 μ M/ml. "Bulk protein" is the TCA- and 75% ethanol/0.18 N HCl-insoluble material. The purification of insulin included the final step of chromatography on CM-cellulose, which yields insulin devoid of any detectable impurities.¹⁷

for 1 hr at 17°C, essentially the same pattern was obtained. This lag period of 30–60 min is probably due to the time needed for the amino acid in the buffer to mix with the extracellular and intracellular amino acid pool of the minces.²⁴ After incubation for 1 hr or longer, proline incorporation into insulin is consistently higher than into bulk protein.

The effect of puromycin on amino acid incorporation is shown in Figure 2. Addition of 50 µg/ml (10^{-4} M) puromycin inhibited amino acid incorporation into bulk protein by 84 per cent and into insulin by 81 per cent.

In a first attempt to produce a gradient of radioactivity along the insulin chains, two batches of islet tissue minces were incubated for 30 min with proline- H^3 and for 5 hr with proline- C^{14} , as internal standard. When the three proline-containing peptides, rather than the isolated prolines, were counted, the amount of H^3 -activity appeared to parallel the size of the counted peptide. Analysis of the proline- H^3 used in this experiment showed radiochemical impurities in the amount of 14 per cent, mostly in the form of aspartic acid. In the following experiments, therefore, the peptide-derived proline residues were isolated and counted, as outlined in the *Experimental Procedures* section.

The results of two experiments comparing the specific activities of the three isolated proline residues in insulin are shown in Table 1. In both experiments, the specific C^{14} -activities of the three prolines are not significantly different, whereas the specific H^3 -activities are markedly different. Hence, incubation for 8 hr led to uniform labeling of all three proline residues in insulin, whereas incubation for 30 or 15 min led to uneven labeling. The difference in the labeling pattern due to differences in time of incubation is even more evident from the H^3/C^{14} ratios which are independent of the actual amounts of proline counted. In both experiments, the H^3/C^{14} ratios of the three proline residues are increasing in the order $B_2 < A < B_{28}$ (1:1.56:2.70 in experiment I, and 1:1.85:2.94 in experiment II).

Discussion.—The short length of the two insulin chains raises the possibility that these polypeptides might be assembled by mechanisms similar to the ones which serve for the synthesis of short bacterial polypeptides like tyrocidine.²⁵ Our experimental results show, however, that insulin is synthesized like protein, and unlike tyrocidine, for the following two reasons: (1) Puromycin, an inhibitor of protein synthesis, strongly inhibits amino acid incorporation into insulin as well as into bulk protein, while the synthesis of tyrocidine is not affected by inhibitors of pro-

TABLE 1
INCORPORATION OF H^3 -PROLINE AND C^{14} -PROLINE INTO THE THREE PROLINE RESIDUES
OF ANGLERFISH INSULIN

	Experiment 1 H ³ -proline 30 min C ¹⁴ -proline 8 hr			Experiment 2 H ³ -proline 15 min C ¹⁴ -proline 8 hr		
	dpm H ³ / 0.2 µM*	dpm C ¹⁴ / 0.2 µM*	H ³ /C ¹⁴	dpm H ³ / 0.2 µM†	dpm C ¹⁴ / 0.2 µM†	H ³ /C ¹⁴
Pro A	8,320	7,250	1.15	1,184	2,010	0.59
Pro B ₂	6,430	7,920	0.81	740	2,290	0.32
Pro B ₂₈	16,550	7,550	2.19	2,321	2,460	0.94

* Specific activities expressed as dpm/0.2 µM of original peptide as determined with the amino acid analyzer.

† Specific activities expressed as dpm/0.2 µM of isolated proline as determined by the ninhydrin reaction of an aliquot of the sample to be counted.

800 mg wet weight of islet tissue minces were incubated for 30 (Expt. 1) or 15 (Expt. 2) min in Krebs-Ringer bicarbonate buffer (pH 7.4) with H^3 -proline, 5.0 mC/µM, 0.5 µM/ml. 200 mg wet weight of islet tissue minces were incubated for 8 hr in Krebs-Ringer bicarbonate buffer (pH 7.4) with C^{14} -proline, 200 µC/µM (Expt. 1) or 50 µC/µM (Expt. 2), 0.5 µM/ml. In both experiments, H^3 - and C^{14} -labeled proteins were combined after incubation and processed together.

tein synthesis.²⁵ (2) Short time incubation with proline- H^3 (30 and 15 min at $17^\circ C$ correspond to ~ 7.5 and 3 min, respectively, at $37^\circ C$) yielded nonuniformly labeled insulin. Since the H^3/C^{14} ratio of proline B_2 , which is near the N-terminal end, is lower by a factor of 2.7 and 2.94, respectively, than the H^3/C^{14} ratio of proline B_{28} near the C-terminal end of the B-chain, it is concluded that biosynthesis of insulin B chain starts at its N-terminal end. This conclusion is independent of the final answer to the question whether insulin is synthesized as two separate chains or as a single-chain precursor, but the results are in keeping with recent results in the biosynthesis of other proteins,⁵⁻⁹ and represent, therefore, further evidence that insulin is synthesized by mechanisms similar or identical to the ones operative in protein synthesis.

Kritzman *et al.*²⁶ have described uneven labeling of insulin by mere incubation of labeled phenylalanine with beef insulin. This type of incorporation, whatever its mechanism, could contribute to uneven labeling obtained with living tissue, but, in this case, the labeling should remain uneven even after long times of incubation. This was not the case in the present experiments. Hence, the type of amino acid replacement described by Kritzman *et al.* can be ruled out as a contributing factor to the results here recorded.

The fact that incubation with proline- C^{14} for 8 hr yielded uniformly labeled insulin is consistent with the assumption that islet tissue *in vitro* is able to synthesize new insulin chains, and not to complete only chains initiated *in vivo*. The fact that the

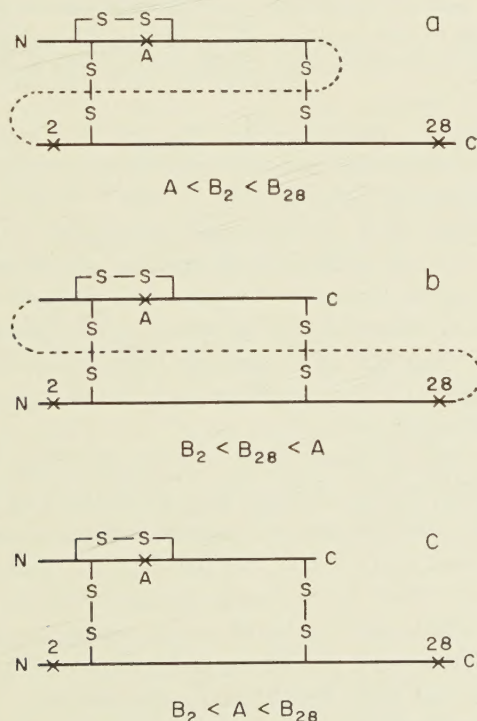


FIG. 3.—Expected labeling pattern of the three proline residues in *Lophius* insulin if (a) + (b) a single-chain precursor of insulin is assumed; (c) separate synthesis of insulin chains is assumed. See text for explanations.

time course of incorporation of radioactive proline into insulin remains linear over 8 hr further supports this assumption. Uniform labeling after prolonged incubation with labeled amino acids was also obtained in globin chains,²⁷ egg white lysozyme,⁸ and ribonuclease.⁹ An earlier report by Vaughan and Anfinsen,²⁸ however, describes uneven labeling of insulin and ribonuclease after incubation for 4 hr. No explanation has been offered for this discrepancy.⁹

As to the question put forward in the introduction, whether insulin is synthesized as two separate chains or as a single-chain precursor, three alternatives expressed in three different labeling patterns can be envisaged: (a) synthesis of a single-chain precursor starting at the N-terminal end of the A chain—in this case proline A is expected to have the lowest specific activity of all three prolines (Fig. 3a); (b) synthesis of a single-chain precursor starting at the N-terminal end of the B chain—in this case proline A is expected to have the highest specific activity (Fig. 3b); (c) separate synthesis of the two insulin chains—in this case proline A is expected to have a specific activity intermediate between the activities of prolines B₂ and B₂₈.

Patterns (a) and (b) (specific activity of proline A either lowest or highest) are required for the existence of a single-chain precursor, but do not prove it, for similar results can be produced by alternative (c), i.e., separate synthesis of the two chains, if the chains are synthesized at different rates, or the pools of finished A and B chains were of different sizes. Hence, experimental results in agreement with patterns (a) and (b) would be inconclusive.

While no labeling pattern could have excluded separate synthesis of the two chains, the observed data [pattern (c)] do eliminate the possibility of single-chain assembly. Hence, we can come to the conclusion that the two chains of insulin are synthesized as separate chains.

This still leaves open the question of how the two chains once finished combine *in vivo*, since, as mentioned earlier, reconstitution of active insulin from the two reduced chains takes place with very low yield *in vitro*.⁴ It is of course possible that this low yield is a consequence of inadequate techniques. Du *et al.*²⁹ and Jiang *et al.*³⁰ claim to have obtained yields up to 20 per cent and 50 per cent, respectively. Since free chains have never been found in pancreas, chain-to-chain linkage *in vivo* must be rather efficient, suggesting the presence, in islet tissue, of an enzyme accelerating the rate of oxidation or of disulfide interchange in insulin. A similar enzyme for ribonuclease and lysozyme has been found in rat liver microsomes³¹ and in pigeon and chicken pancreas.³²

The finding of separate synthesis of the two insulin chains suggests that there may be cases of relative overproduction, with consecutive accumulation, of either chain, analogous to the accumulation of L-chains in Bence-Jones proteins.³³

Summary.—Islet tissue minces from the fish *Lophius piscatorius* have been incubated with proline-C¹⁴ for 8 hr and with proline-H³ for 15 or 30 min at 17°C. After incubation, the minces were combined, insulin was extracted, purified, and separated into three peptides, each containing one proline residue. The three proline residues were isolated and their H³/C¹⁴ ratios determined. From these radioactivity values and from knowledge of the positions of proline in the chains, evidence was obtained for the conclusion that the two constituent chains of insulin are synthesized separately.

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¹ There is a third, intrachain disulfide bridge on the A chain.

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Isolation and Partial Structural Analysis
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Isolation and Partial Structural Analysis of Insulin from the Separate Islet Tissue of *Lophius piscatorius**

René E. Humbel† and Arthur M. Crestfield

ABSTRACT: The isolation and partial structural analysis of pure insulin from anglerfish (*Lophius piscatorius*) was undertaken as a basis for studies on the biosynthesis of insulin in the separate islet tissue of this fish. A procedure is described for isolation of the insulin, first by gel filtration and then by chromatography on CM-cellulose, with a recovery of about 67% and a yield of pure insulin of about 3 mg/g of wet tissue. A new method for separation of the A and B chains following reduction and carboxymethylation is presented. Amino acid

analyses show that the two chains contain the same number of residues as does insulin from beef. The A chain contains one residue each of proline and arginine and has COOH-terminal asparagine (or glutamine). Trypsin does not hydrolyze the arginyl bond in this chain. The B chain contains two residues of proline. The NH₂-terminal sequence is valyl-alanyl-prolyl and that at the COOH terminus is -prolyl-lysine. A procedure is presented for isolating the three residues of proline in three separate peptides.

The endocrine pancreatic tissue of the anglerfish (*Lophius piscatorius*) is in the form of an easily separable and relatively large organ, the Brockmann body (Bargmann, 1939). This tissue might be well suited for studies on the biosynthesis of insulin since the amount of extraneous protein to be removed would be less than with other species. Therefore the isolation and study of the partial structure of the insulin from this fish was undertaken. An earlier report dealt only with the separation of the insulin (Humbel, 1963). Although that insulin preparation was highly active (23 units/mg) and was homogeneous by paper electrophoresis, amino acid analyses later showed that it was impure.

This paper describes the purification of the insulin from anglerfish, the amino acid composition of the separated reduced and carboxymethylated (RCM)¹ chains, their NH₂- and COOH-terminal residues, and the amino acid composition of certain peptides formed by tryptic and chymotryptic hydrolyses. This information is sufficient to serve as a basis for the work on the biosynthesis of insulin described elsewhere (Humbel, 1965).

Experimental Methods

Materials. Sephadex (G-25, G-50, and G-75, medium grade, from Pharmacia, Uppsala) was washed and de-aerated as described by Crestfield *et al.* (1962). CM-cellulose (medium grade, capacity 0.8 meq/g, from Carl

Schleicher & Schuell Co., lot 1317) was washed according to Peterson and Sober (1962). Dowex 50-X2 (200–400 mesh, in the H⁺ form, the Baker Chemical Co., lot 22051) was washed according to Moore and Stein (1951). Urea was deionized (Benesch *et al.*, 1955) prior to use by passing a 10 M solution at room temperature through a mixed-bed resin column (Amberlite MB-1, Mallinckrodt). Iodoacetic acid (Distillation Products, Inc.) was recrystallized twice from petroleum ether. Crystalline Zn-insulin from beef, lot 719106, was kindly provided through the courtesy of Dr. M. Root, Eli Lilly Co. DFP-treated carboxypeptidase A and B (lots CoA-DFP-6127 and CoB-DFP-27), trypsin (twice crystallized, salt free, lot TRL 6246), and chymotrypsin (prepared by ion-exchange chromatography, lot CDC 47) were purchased from the Worthington Biochemical Corp. All other chemicals were analytical grade reagents used without further treatment. Protein concentrations were measured either by their absorbancy at 276 mμ, when column eluates were monitored, or by Kjeldahl digestion and Nesslerization.

Isolation of Insulin from Islet Tissue. Isolated Brockmann bodies from anglerfish were obtained in the frozen state from New England Biological Associates, Narragansett, R. I. The average islet weighs about 70 mg. Islets were cleaned by removal of surrounding fibrous and exocrine pancreatic tissue and by passage through a steel screen. Each gram of strained tissue was homogenized in 10 ml of 10% (w/v) trichloroacetic acid in an all-glass homogenizer. The precipitate was washed three times with 10 ml of 5% (w/v) trichloroacetic acid and then extracted three times with about 7 ml of 74% ethanol, 0.18 N in HCl. Most of the ethanol was removed with a rotary evaporator and a solution of 1 M trisodium citrate was added to bring the aqueous residue to pH 3.5–4.5 according to indicator paper. Lipids were then extracted with 3 volumes of methylene chloride. Residual meth-

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¹ Abbreviation used in this work: RCM, reduced and carboxymethylated.

ylene chloride was removed *in vacuo* and a solution of 100% (w/v) trichloroacetic acid was added to a concentration of 5% (w/v). The precipitate which formed on standing for 2 hours at 4° was collected by centrifugation and taken up in 2 ml of 5 M acetic acid, 0.15 M in NaCl. Insoluble proteins were removed by centrifugation and the turbid supernatant liquid was applied to a column (50 × 2 cm) of Sephadex G-75 which had been equilibrated with the same solvent. The pooled fractions which contained the insulin were concentrated by rotary evaporation to a volume of about 2 ml and then desalted by passage through a column (30 × 0.9 cm) of Sephadex G-25 equilibrated with 50% (v/v) aqueous acetic acid.

Chromatography on columns of CM-cellulose was used for the final purification. Essentially, the method of Yamamoto *et al.* (1960) for separation of bonito insulin I and II was followed. The sample of desalted insulin was concentrated in an evacuated desiccator over NaOH pellets until 5–20 mg of protein was in 0.1–0.2 ml of approximately 50% acetic acid, 1–2 ml of 0.5 M sodium acetate buffer of pH 4.0 was added, and the solution was applied to a column (30 × 0.9 cm) of CM-cellulose which was equilibrated with the same buffer. Elution was at 20 ml/hr from a mixing chamber of 100-ml capacity which was initially filled with a solution 0.2 M in formic acid and 0.4 M in acetic acid and was fed from a reservoir of 1.2 M formic acid.

Biological assays were carried out by Dr. J. Steinke, Department of Medicine, Harvard Medical School, according to the *in vitro* method of Renold *et al.* (1960). An exactly determined amount of insulin in 50% acetic acid was dried *in vacuo* over NaOH at ambient temperature. For the assay, the insulin was dissolved in a small amount of 0.05 N HCl and further diluted with the buffer used for the adipose tissue assay.

Reduction and Alkylation of Insulin. The reduction and carboxymethylation of insulin was achieved by the procedure described by Crestfield *et al.* (1963) for 5–50 mg of protein, except that one-third or one-sixth of the quantities of reagents was used and the final volume was 4 or 2 ml. The RCM insulin chains were separated from reagents by passing the mixture over a column of Sephadex G-25 (40 × 2 cm or 40 × 1 cm), wrapped in aluminum foil, and equilibrated with 50% acetic acid. Elution of the peptide mixture was monitored by absorption measurements at 276 mμ. The pooled eluate was concentrated in a rotary evaporator at 25° and finally dried in an evacuated desiccator over NaOH pellets at room temperature.

Separation of RCM Chains of Insulin. The RCM A and B chains of insulin were separated on a column of Dowex 50-X2 (12 × 0.9 cm) which was equilibrated with 0.2 M ammonium acetate-acetic acid buffer, pH 4.5. The mixture of chains was applied to the column in 1 ml of 0.2 M ammonium acetate buffer to which had been added solid urea to 6 M concentration and formic acid to pH 4.5. The RCM A chain was eluted with this same buffer at a flow rate of 0.4 ml/min. As soon as the A chain was eluted, 5 ml of buffer without urea was passed through the column to rinse out the bulk of the urea,

and then the RCM B chain was eluted with 1 M ammonium hydroxide. The contact of B chain with ammonium hydroxide was as short as possible, usually about 20 minutes. The eluted B chain was rapidly concentrated in a rotary evaporator at room temperature. Glacial acetic acid was added to make the solution 50% in acetic acid and the solution was further concentrated to about 2 ml. To free the preparations of the A and B chains from residual reagents the two preparations were passed through a column (30 × 0.9 cm) of Sephadex G-25 in 50% acetic acid.

Amino Acid Analyses. Hydrolysis of peptides was performed as described by Crestfield *et al.* (1963) with careful evacuation and deaeration of the hydrolysis tubes. The hydrolysate was dried rapidly in a flash evaporator, dissolved in citrate buffer (pH 2.2), and analyzed by means of a Spingo automatic amino acid analyzer according to the procedure described by Spackman *et al.* (1958). More recent analyses were done with an accelerated procedure (Spackman, 1963), a sample injector (Crestfield, 1963), and an experimental photometer ten times more sensitive² than the one used in the regular instrument.

The spectrophotometric estimation of the tyrosine-tryptophan ratios in insulin preparations was carried out by the method of Goodwin and Morton as described by Beaven and Holiday (1952).

Enzymic Hydrolyses. For digestion by carboxypeptidase A and B, 0.05 μmole of peptide was dissolved in 45 μl of 0.1 M sodium bicarbonate, pH 7.6 (for carboxypeptidase A), or 45 μl of 0.1 M phosphate buffer, pH 7.6 (for carboxypeptidase B). Enzyme (25 μg) in 5 μl of buffer was added; the substrate-enzyme ratio was 70:1. The mixture was incubated at 23° for 2 hours. The reaction was stopped by addition of 1.15 ml of citrate buffer, pH 2.2, and the sample was analyzed on the 60- and 15-cm columns of the amino acid analyzer.

For digestion by trypsin and chymotrypsin, the enzyme was dissolved in 0.001 N HCl to give a 0.16% solution; 2 or 4 μl, respectively, was added to 0.1 μmole of peptide in 25 μl of 0.2 M ammonium bicarbonate, pH 8.1. Substrate-enzyme ratios were about 100:1 and 50:1 (w/w), respectively. Reactions were allowed to proceed at 23° for 90 minutes.

Electrophoretic Fractionation of Enzymic Digests. The enzymic hydrolysates were applied to dry strips of Whatman 3MM paper (6 × 45 cm) which had been soaked overnight in 50% acetic acid, and then hardened with 95% ethanol and dried in air as described by Crestfield and Allen (1957). The spots were allowed to dry between multiple applications. The strip was then thoroughly wetted from both ends with 0.2 M ammonium bicarbonate, pH 8.1, placed in the apparatus described by Crestfield and Allen (1955), and subjected to a potential of 800 v (30 ma) for 2–4 hours. After the strips were dried on the heated plate of the apparatus,

² A cell of 20-mm light path, like the one described by Crestfield (1963), replaced the 2-mm cell. Cadmium sulfide photoconductive cells (Clairex 607L-167) with batteries were used instead of the usual photovoltaic cells.

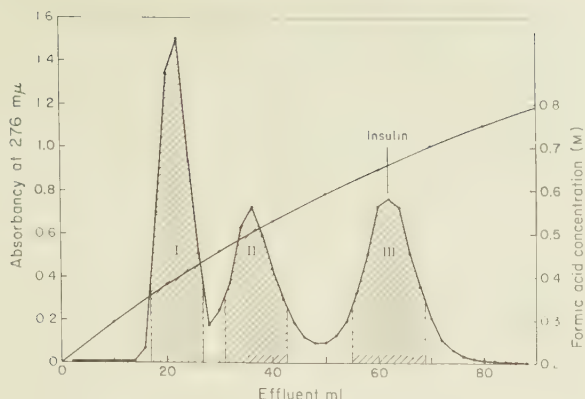


FIGURE 1: Separation of insulin from two small polypeptides by means of CM-cellulose. Protein (5–20 mg) ($30 D_{276\text{m}\mu}^{1\text{cm}}$ units in this case) in 1–2 ml of 0.5 N sodium acetate, acetic acid buffer of pH 4 applied to a column (30×0.9 cm) which was equilibrated with the same buffer. Elution at 20 ml/hr from a closed mixing flask of 100-ml capacity began with a solution 0.2 M in formic acid and 0.4 M in acetic acid. The reservoir contained 1.2 M formic acid. Peak III is pure insulin, obtained in a yield of about 3 mg/g of wet tissue.

they were kept for an additional 0.5 hour in an oven at 80° . The peptides were localized on a duplicate channel on the same strip by dipping in a solution of 95% alcohol containing 0.5% ninhydrin. Corresponding rectangular areas from the unstained sheet of paper were cut out and one side was trimmed to form a triangular tip. They were developed chromatographically in ascending fashion in 50% acetic acid to concentrate the peptide into the uppermost tip of paper (see Crestfield and Allen, 1956). After 2 hours the tips were dried, held in forceps, cut off from the bulk of the paper piece, and placed in 1 ml of 50% acetic acid in special hydrolysis tubes. After an additional 2 hours the paper tips were removed, the acetic acid was evaporated in a rotary evaporator, and 6 N HCl was added to the tubes for hydrolysis to amino acids.

NH_2 - and COOH-Terminal Residues by Chemical Methods. Edman degradation: The modification by Guidotti *et al.* (1962) was used and the released NH_2 -terminal residue was determined by the subtractive method of analyzing the residual peptide. Hydrazinolysis was kindly performed by Mr. M. Ruttenberg, by the method of Akabori *et al.* (1952).

Results and Discussion

Chromatography by the procedure of Yamamoto *et al.* (1960) of that insulin preparation from anglerfish which is obtained by gel filtration in 30% acetic acid (Humbel, 1963) yields three peaks as shown in Figure 1. Fractions I and II contained tryptophan and methionine, and in amino acid composition resembled glucagon, as isolated from pork (Behrens and Bromer, 1958). These fractions also resembled glucagon in size, as

TABLE I: Amino Acid Composition of the A and B Chains of RCM Insulin from Anglerfish.

Amino Acid	RCM Insulin ^a			
	A Chain ^b		B Chain ^b	
	Found	Nearest Integer	Found	Nearest Integer
Aspartic acid	4.4	4	3.1	3
Glutamic acid	3.0	3	1.1	1
Glycine	1.0	1	3.0	3
Alanine	0	0	3.0	3
Valine	0.5 ^c	1	2.9	3
Leucine	1.0	1	4.0	4
Isoleucine	1.3 ^c	2	0	0
Serine ^d	0	0	0.9	1
Threonine	0	0	0	0
Methionine	0	0	0	0
S-Carboxymethyl-cysteine	4.1	4	2.0	2
Proline	1.0	1	2.1	2
Phenylalanine	0.9	1	2.0	2
Tyrosine ^d	1.0	1	2.0	2
Lysine	0	0	1.0	1
Arginine	1.0	1	1.0	1
Histidine	1.0	1	2.0	2
Ammonia ^e	4.4	4	2.1	2
Tryptophan ^f	0	0	0	0
Total number of residues		21		30

^a Each column represents the results of a single analysis by ion-exchange chromatography of a 22-hour hydrolysate. The results are expressed as the number of amino acid residues per molecule, assuming there is one residue of arginine in each chain. ^b Amounts of amino acids less than $0.001 \mu\text{mole}$ are called zero. At the loads used with the special photometer this amount represents less than 0.1 residue. ^c The isoleucyl-valyl bond is only partly hydrolyzed in 22 hours with beef insulin. ^d Not corrected for loss during hydrolysis. ^e Amide NH_3 is that remaining after correction for free NH_3 in the intact peptide preparation and for adventitious NH_3 produced by acid hydrolysis from reagents and glassware. ^f Tryptophan was determined spectrophotometrically (Beaven and Holiday, 1952).

indicated by their retention volumes on this column, and they showed less than 0.5% of the biological activity of insulin. Fraction III is pure insulin which showed in two sets of triplicate determinations a biological potency of 26 and 27 units/mg with an index of precision of 0.1008, and 95% confidence limits of 18–33 and 20–37, respectively. Different preparations of beef insulin show activities in the range of 22–27 units/mg. The total yield of pure insulin was about 3.2 mg/g of wet tissue, cor-

TABLE II: Amino Acid Composition of Tryptic and Chymotryptic Peptides from the RCM B Chain of Insulin.^a

Amino Acid	Peptide ^b							
	Bt ₁		Bt ₂		Bt ₁ c ₁		Bt ₁ c ₂	
	Found	Theory	Found	Theory	Found	Theory	Found	Theory
Aspartic acid	2.0	2	1.0	1	0	0	2.3	2
Glutamic acid	1.1	1	0	0	1.0	1	0	0
Glycine	2.0	2	1.0	1	0	0	2.4	2
Alanine	3.0	3	0	0	2.0	2	1.4	1
Valine	2.9	3	0	0	1.1	1	1.9	2
Leucine	4.1	4	0	0	1.0	1	3.1	3
Serine ^c	0.7	1	0	0	0	0	0.8	1
S-Carboxymethyl-cysteine	2.0	2	0	0	0	0	1.3	2
Proline	1.0	1	1.0	1	1.0	1	0	0
Phenylalanine	0	0	1.9	2	0	0	0	0
Tyrosine ^c	0.9	1	0.9	1	0	0	0.7	1
Lysine	0	0	1.0	1			0	0
Arginine	1.0	1	0	0			1.0	1
Histidine	1.9	2	0	0			0.9	1

^a These results are from 22-hour hydrolysates. The numbers are expressed as the number of amino acid residues per molecule, assuming there to be 4, 1, or 3 residues of leucine in peptides Bt₁, Bt₁c₁, and Bt₁c₂, respectively, and assuming there to be 1 residue of glycine in peptide Bt₂. ^{b,c} See footnotes *b* and *d*, Table I.

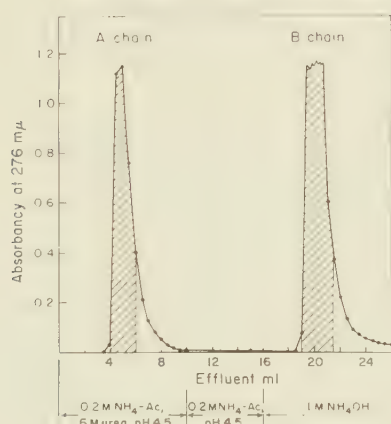


FIGURE 2: Isolation of RCM A chain and RCM B chain from anglerfish insulin by chromatography on Dowex 50-X2. RCM protein (10 mg) was dissolved in 1 ml of eluent and applied to a column of Dowex 50-X2 (12 × 0.9 cm). Rate of flow was 0.4 ml/min for the first buffer and 0.6 ml/min for the 1 M ammonia.

responding to a recovery of about 64% based upon bioassays of homogenates of islet tissue.

The methods of separation of the A and B chains from RCM insulin of beef which was described by Crestfield *et al.* (1963) could not be applied to this insulin since separation was unsatisfactory. Small-scale tests showed that at pH 4.5 neither chain would dissolve in the absence of urea, whereas at about pH 8 the B chain did dissolve without addition of

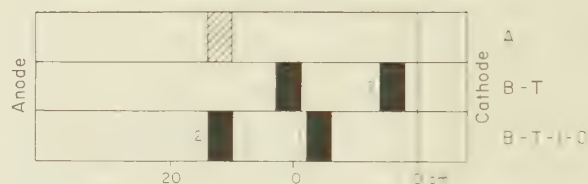


FIGURE 3: Paper electrophoretic separation of the RCM A chain and of tryptic and chymotryptic hydrolysates of the RCM B chain of insulin. NH_4HCO_3 (0.2 M) was used on Whatman 3MM paper. Peptides were detected with ninhydrin. Each sample was separated in 3 hours at 800 v across paper of 45 cm length.

urea. Therefore a convenient preparative procedure was devised in which Dowex 50-X2 is employed, first with a buffer containing urea to elute the A chain and then rapidly with 1 M ammonia to elute the B chain. A typical separation is shown in Figure 2. The separated chains were pure, as seen in Table I from the absence of lysine in the A chain and the absence of isoleucine in the B chain. The total number of residues is 21 in the A chain and 30 in the B chain. These values are the same as for beef insulin.

The two chains yielded the following results upon application of the carboxypeptidase and Edman procedures for NH_2 - and COOH -terminal residues: *RCM A chain*: Carboxypeptidase A released 0.92 residue of asparagine (or glutamine) and traces of CM-cysteine, tyrosine, and aspartic acid. The COOH -terminal residue is therefore likely to be asparagine, since all other insulins studied thus far have this amino acid in that

position.³ *RCM B chain*: Carboxypeptidase A showed no reaction. Carboxypeptidase B released 0.76 residue of lysine, showing that the COOH terminus is lysine, which in other insulins is usually found second from this end. Edman degradation established the NH₂-terminal sequence as valyl-alanyl-prolyl with the following analyses:

	Val	Ala	Pro
B chain	2.92	2.90	2.00
Stage 1	<u>1.96</u>	2.82	1.69
Stage 2	1.90	<u>2.07</u>	1.80
Stage 3	1.95	1.90	<u>1.19</u>

Tryptic and chymotryptic hydrolyses of the two chains were carried out in order to localize, approximately, the 3 residues of proline, since it was incorporation of this amino acid which was to be studied in the biosynthesis of insulin. Only very limited amounts of the peptides were available, but the added sensitivity of the experimental amino acid analyzer encouraged a trial of paper electrophoresis for isolation of the enzymically produced fragments. Trypsin failed to hydrolyze the arginyl bond in the A chain. This is found to be the case also with other fish insulin (Kotaki, 1963). Tryptic hydrolysis of the B chain yielded two peptides, as seen in Figure 3. The analyses shown in Table II identify these as a peptide (Bt₁) containing 23 residues, including 1 residue of arginine, and a peptide (Bt₂) containing 7 residues, among them 1 proline and the single lysine residue from the COOH terminus of the B chain. Chymotryptic hydrolysis of peptide Bt₁ yielded the two peptides shown in Figure 3. Their analyses shown in Table II identify them as a peptide (Bt_{1c1}) containing 6 residues, including proline 3, near the NH₂ terminus of the B chain and a peptide (Bt_{1c2}) of 16 residues. In order to establish the position of the second proline in the B chain, the COOH-terminal lysine was removed from peptide Bt₂ by carboxypeptidase B. The remaining hexapeptide was then subjected to hydrazinolysis. Proline was found in 30% yield, thereby establishing it to be residue 29, adjacent to the terminal lysine. Hence, by a combination of chromatographic and electrophoretic procedures, three peptides

are easily resolved, each one containing a different one of the 3 residues of proline in this insulin.

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³ L. F. Smith, personal communication.

